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Study of Toxic and Antigenic Structures of Botulinum Neurotoxins

Annual Report

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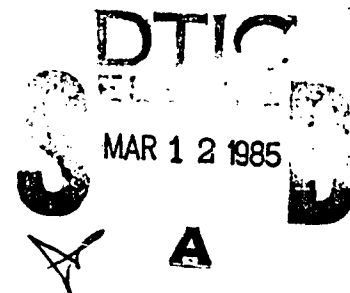
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The immunologically distinct botulinum neurotoxin (NT) types A, B, E and F were defined in terms of amino acid composition (number of residues/protein molecule). The subunit heavy and light chains of type A NT were separated and their amino sequences were partially determined. Histidine residues of type A and E appear critical for toxicity but not for serological reactivity. The paralyzing effect of type B has a shorter duration of action, than type A, at the rat neuromuscular junction but less effectively antagonized by 3,4-diaminopyridine.		

Summary Page

The two broad goals of our research are to 1) determine the precise structure of botulinum neurotoxin (NT), and 2) establish the relationship between the structure and biological activities of the NT (namely its neurotoxicity and antigenicity). We have also initiated studies on the mechanism of its action.

I. Structure:

Amino Acid Composition: We have completed a rigorous examination of the amino acid composition of the NT types A, B, E and F. Each neurotoxin type was purified in three separate batches. When two methods for purification of one type were available (e.g., type A, B and F) two batches of one type were purified by the same method and one was purified by a different method, and the three batches were analyzed separately. Each of the three neurotoxin types (A, B and F) purified by two methods was comparable in purity and similar in amino acid composition.

The immunologically distinct botulinum NT types A, B, E and F are now defined in terms of amino acid composition. This allows, for the first time, a chemical basis to compare the NT types. Similarity between types A and E is closer than between any other two types. Five amino acids do not differ by more than 1 residue; e.g., number of residues in Type A/Type E were Thr 75/75; Pro 44/45; Tyr 71/70; His 14/15; Trp 17/16. The next best resemblance by this criterion is between types A and B; types A and F; as well as types E and C. In each pair 3 amino acids do not differ by more than 1 residue.

Amino Acid Sequence: The subunit heavy and light chains of type A NT were separated and purified by ion exchange chromatography. The sequence of the first 9 and 16 amino acid residues of the N-terminal end of these two chains were established. Also the first 4 residues of the single chain type E were determined.

II. Structure-Function Relationship:

Selective modification of amino acid residues, to search "active sites": Role of histidine residues: Type A and E NT were modified with diethylpyrocarbonate (ethoxyformic anhydride), a reagent highly specific for histidine residues. Type E could be completely detoxified without causing detectable damage to its serological reactivity. Under identical modification reaction conditions, type A was incompletely detoxified with some alteration in its serological reactivity. The completely detoxified type E NT, used as toxoid, elicited antibodies in rabbits. The antiserum precipitated and neutralized the NT. This toxoid, considered as a second generation toxoid (first generation toxoid being a crude preparation of NT detoxified with formalin) has the following noteworthy features: i) prepared with pure NT, it is ii) electrophoretically homogenous, unlike pure NT detoxified with formaldehyde, and iii) its chemical modification is more specific than the products that form over 7-10 days of reaction between protein and formaldehyde.

III. Mechanism of Action:

We have found different effects of types A and B botulinum NT on transmitter release at the rat neuromuscular junction. Both toxins decrease the frequency of miniature endplate potentials but type A did so to a greater extent. For example, at 3 days after toxin injection nerve-impulse-evoked transmitter release was reduced more for type A treated muscles than for type B. However, 3,4-diaminopyridine, an agent which increases nerve-evoked transmitter release by increasing Ca^{++} influx, was more effective in reversing the paralysis in type A than in type B-treated muscles. The results show that type B NT differs from type A, mainly by a shorter duration of action and by being less effectively antagonized by 3,4-diaminopyridine. These findings have implications for the medical treatment of the types A and B botulism.

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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1. Defining botulinum neurotoxin type A, B, E and F in terms of amino acid composition: To develop reliable data on the amino acid composition of botulinum neurotoxin types A, B, E and F we had planned to analyze three batches of each of the neurotoxin types. Each batch of a neurotoxin type was to be isolated from a separate toxin producing bacterial culture (using an inoculum from two laboratories, if available), and the neurotoxin was to be purified by at least two different methods, if available. Compared to triplicate analysis of a single batch of a neurotoxin type our approach is a more rigorous examination of (i) the amino acid composition of the neurotoxin, (ii) consistency of the purified neurotoxin, independent of source of bacterial culture inoculum and method of purification.

Type A: This study, most of which was done before March 1983, was completed after March 1983 and a manuscript was submitted for publication on September 23, 1983 (see list of publications, #12).

Type B: This study initiated before March 1983 was completed recently. A manuscript was submitted for publication on September 9, 1983 (see list of publications, #11).

Type E: This study was completed and submitted for publication before March 1983. It has appeared in print (see list of publications, #4).

Type F: This study was completed and submitted for publication before March 1983. It has appeared in print (see list of publications, #5).

2. The heavy (H, mol. wt. 97,000) and light (L, mol. wt. 53,000) chains of type A neurotoxin (mol. wt. 145,000) were separated according to a published method following reductive cleavage of the -S-S- bond(s) holding them together. But the isolated chains, particularly the H chain, remains contaminated with the dichain and single chain neurotoxin. (The purified type A neurotoxin is a mixture of nicked and unnicked molecules of identical mol. wt.). We developed a procedure to isolate the H chain from its unreduced parent dichain and single chain neurotoxin.

3. The isolated H- and L-chains of type A were subjected to amino acid sequence analysis (in collaboration with Dr. James Schmidt of USAMRIID). We now know the sequence of the first 9 and 16 amino acid residues of the N terminal end of H- and L-chains respectively. This information was presented at the annual meeting (June 1983) of American Society of Biological Chemists (see list of publications, #10).

4. We (in collaboration with Dr. James Schmidt, USAMRIID) have also determined the sequence of the first 4 amino acid residues of the N terminal end of the single chain type E neurotoxin (see list of publications, #10).

The amino acid sequence studies demonstrated convincingly the high degree of purity of the type E neurotoxin and the two chains of type A as prepared in our laboratory. Until now "single band" in PAGE-SDS was our best criteria of purity and we believed that a single band was not a composite of two

unresolved bands. Our trust in the resolving power of PAGE-SDS was not misplaced.

5. Role of histidine residues - the toxicity and antigenicity was further studied. Our preliminary observation was confirmed and extended: Selective modification of histidine residues destroys toxicity of type E neurotoxin completely (mice survived beyond 4 days post i.v. and i.p. injection) but toxicity of type A neurotoxin was not completely lost. The completely detoxified type E neurotoxin does not show loss in its serological reactivity, whereas incompletely detoxified type A neurotoxin appears to show some loss in serological reactivity. These observations were presented at the annual meeting (April 1983) of American Society of Microbiology (see list of publications, #9).

6. Second generation immunogen (toxoid): The type E neurotoxin completely detoxified by histidine modification appears to have the potential to be used as toxoid. Rabbits immunized with such a preparation produce antiserum that neutralizes the toxin. This study was completed and submitted for publication after March 1983 (see list of publications, #9 and 13).

7. Through tryptic digestion and chromatography we have isolated a large fragment of the type A neurotoxin. The fragment (mol. wt. ~100,000) is composed of the L chain (mol. wt. 53,000) and approximately one half of the H chain, the two being linked by a -S-S- bond (probably the -S-S- that holds the L and H chain in the dichain neurotoxin of 145,000 mol. wt.). The fragment of the H chain was also isolated in essentially pure form (PAGE-SDS). It is of mol. wt. ~47,000 and serologically reactive to the antineurotoxin serum (reaction of partial identity in Ouchterlony plate). Therefore at least one antigenic determinant of the neurotoxin appears to be located on this half of the H chain.

8. Collaborative work with Dr. L. L. Simpson (Columbia University) on pharmacology of type E neurotoxin has been published (see list of publications, #6).

9. Collaborative work with Dr. L. Sellin (USAMRIID) on electrophysiology of types A, B and E neurotoxins. Two manuscripts have been submitted; both have appeared in print (see list of publications, #7 and 8).

10. Collaborative work with Maj. Martin Crumrine on immunological aspects. We supplied pure neurotoxin preparations; most recently (Jan. 17, 1983) approximately 13 mg of highly purified type B neurotoxin.

11. Work other than experimental work: Attended (May 24-26, 1982) the Ad Hoc Review of the Botulinum Research Progress currently in progress at USAMRIID; presented our progress report and developed plans for collaborative work, e.g., amino acid sequence studies with Dr. James Schmidt.

An application (research proposal) for a new contract "Exploration of toxic site and bind site of botulinum neurotoxin" was prepared and submitted. The contract has been funded; DAMD 17-83-C-3034, February 1, 1983-January 31, 1985.

An application (funding of new instruments) was prepared and submitted to DOD-University Research Instrumentation Program for three instruments (office of Naval Research, Arlington, VA 22217). Award was made (contract #DAAG 29-83-G-0063).

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